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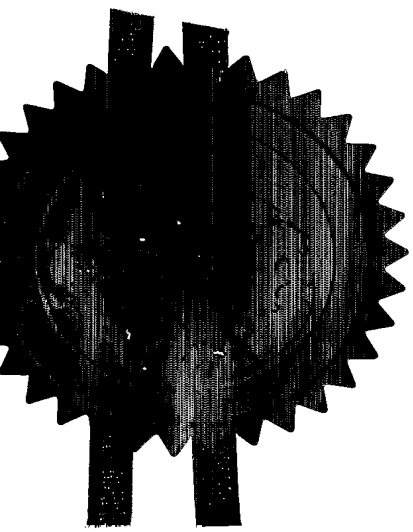

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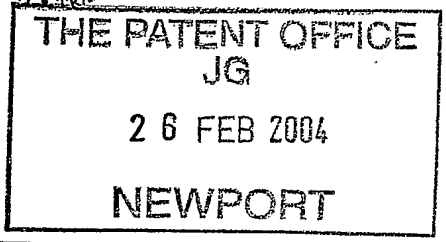

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Patents ADP number *(if you know it)* 8816928001

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4. Title of the invention

Novel Therapies

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Novel Therapies

The present invention relates to methods for inhibiting angiogenesis, as well as to the use of certain reagents in the preparation of medicaments for inhibiting angiogenesis.

Angiogenesis is the process of forming new blood vessels. It occurs normally in the human body at specific times in development and growth. For example, the embryo needs a vast network of arteries, veins, and capillaries. A process called *vasculogenesis* creates the primary network of vascular endothelial cells that will become major blood vessels. Later on, angiogenesis remodels this network into the small new blood vessels or capillaries that complete the child's circulatory system.

Proliferation of new blood vessels also takes place in adults. In women, angiogenesis is active a few days each month as new blood vessels form in the lining of the uterus during the menstrual cycle. Also, angiogenesis is necessary for the repair or regeneration of tissue during wound healing.

The vascular endothelial cell rarely divides, unless stimulated by angiogenesis. Angiogenesis is regulated by both *activator* and inhibitor molecules. Normally, the inhibitors predominate, blocking growth. Should a need for new blood vessels arise, angiogenesis activators increase in number and inhibitors decrease thus prompting the growth and division of vascular endothelial cells and, ultimately, the formation of new blood vessels.

Before the 1960s, cancer researchers believed that the blood supply reached tumors simply because pre-existing blood vessels dilated. But later experiments showed that angiogenesis is necessary for cancerous tumors to keep growing and spreading.

Tumor angiogenesis is the proliferation of a network of blood vessels that penetrates into cancerous growths, supplying

nutrients and oxygen and removing waste products. It starts when tumor cells release molecules that send signals to surrounding normal host tissue, activating certain genes and proteins to encourage growth of new blood vessels.

Small activator molecules produced by the cancer cells signal angiogenesis in the surrounding tissue. More than a dozen different proteins, as well as several smaller molecules, have been identified as *"angiogenic"*. Among these are vascular endothelial growth factor *(VEGF)* and basic fibroblast growth factor *(bFGF)*. VEGF and bFGF are produced by many kinds of cancer cells and by certain types of normal cells, too.

VEGF and bFGF are first synthesized inside tumor cells and then secreted into the surrounding tissue. The binding of either VEGF or bFGF to appropriate receptors activates a signalling cascade into the nucleus of the endothelial cells. The nuclear signal ultimately prompts a group of genes to make products needed for new endothelial cell growth.

The activation of endothelial cells by VEGF or bFGF sets in motion a series of steps toward the creation of new blood vessels. First, the activated endothelial cells produce matrix *metalloproteinases (MMPs)*, a special class of degradative enzymes. These enzymes are then released from the endothelial cells into the surrounding tissue. The MMPs break down the *extracellular matrix*—support material that fills the spaces between cells and is made of proteins and polysaccharides. Breakdown of this matrix permits the migration of endothelial cells. As they migrate into the surrounding tissues, activated endothelial cells begin to divide. Soon they organize into hollow tubes that evolve gradually into a mature network of blood vessels.

Although many tumors produce angiogenic molecules such as VEGF and bFGF, their presence is not enough to begin blood vessel growth. For angiogenesis to begin, these activator molecules

must overcome a variety of angiogenesis inhibitors that normally restrain blood vessel growth.

Almost a dozen naturally occurring proteins can inhibit angiogenesis. Among this group of molecules, proteins called *angiostatin, endostatin,* and *thrombospondin* appear to be especially important. A finely tuned balance, between the concentration of angiogenesis inhibitors and of activators such as VEGF and bFGF, determines whether a tumor can induce the growth of new blood vessels. To trigger angiogenesis, the production of activators must increase as the production of inhibitors decreases.

The discovery of angiogenesis inhibitors raises the question of whether such molecules might therapeutically halt or restrain cancer's growth. Researchers have addressed this question in numerous experiments involving animals. In one striking study, mice with several different kinds of cancer were treated with injections of endostatin. After a few cycles of treatment, the initial (primary) tumor formed at the site of the injected cancer cells almost disappeared, and the animals did not develop resistance to the effects of endostatin after repeated usage.

The discovery that angiogenesis inhibitors such as endostatin can restrain the growth of primary tumors raises the possibility that such inhibitors might also be able to slow tumor metastasis.

It has been known for many years that cancer cells originating in a primary tumor can spread to another organ and form tiny, microscopic tumor masses (metastases) that can remain *dormant* for years. A likely explanation for this tumor dormancy is that no angiogenesis occurred, so the small tumor lacked the new blood vessels needed for continued growth.

One possible reason for tumor dormancy may be that some primary tumors secrete the inhibitor angiostatin into the bloodstream, which then circulates throughout the body and inhibits blood

vessel growth at other sites. This could prevent microscopic metastases from growing into visible tumors.

Additional support for the idea that interfering with the process of angiogenesis can restrain tumor growth has come from genetic studies of mice. Scientists have recently created strains of mice that lack two genes, called Id1 and Id3, whose absence hinders angiogenesis. When mouse breast cancer cells are injected into such angiogenesis-deficient mutant mice, there is a small period of tumor growth, but the tumors regress completely after a few weeks, and the mice remain healthy with no signs of cancer. In contrast, normal mice injected with the same breast cancer cells die of cancer within a few weeks.

When lung cancer cells are injected into the same strain of angiogenesis-deficient mutant mice, the results are slightly different. The lung cancer cells do develop into tumors in the mutant, but the tumors grow more slowly than in normal mice and fail to spread (metastasize) to other organs. As a result, the mutant mice live much longer than normal mice injected with the same kinds of lung cancer cells.

As a result, it is now believed that inhibiting angiogenesis can slow down or prevent the growth and spread of cancer cells in humans, and as a result, a large number of angiogenesis inhibitors are currently being tested in cancer patients.

The inhibitors being tested fall into several different categories, depending on their mechanism of action. Some inhibit endothelial cells directly, while others inhibit the angiogenesis signaling cascade or block the ability of endothelial cells to break down the extracellular matrix.

HAMLET (Human α-lactalbumin made lethal to tumor cells) is a molecular complex that induces cell death in tumor cells. Indeed, the effect is selective for tumor cells and some immature cells and healthy, differentiated cells do not undergo cell death in response to HAMLET. This selectivity implies that

HAMLET reaches unique targets in tumor cells, but not in resistant cells.

As used herein, the term "HAMLET" refers to a biologically active complex of α-lactalbumin (which may or may not be human in origin), which is either obtainable by isolation from casein fractions of milk which have been precipitated at pH 4.6, by a combination of anion exchange and gel chromatography as described for example in EP-A-0776214, or by subjecting α-lactalbumin to ion exchange chromatography in the presence of a cofactor from human milk casein, characterized as C18:1 fatty acid as described in WO99/26979. Variants or derivatives of this complex with similar activity are described for example in International Patent Application No. PCT/IB03/01293.

The cellular targets for HAMLET have been examined by a combination of confocal microscopy and subcellular fractionation [Håkansson et al., 1999 Exp Cell Res. 246, 451-60]. HAMLET binds to the cell surface, and enters the cytoplasm where it interacts with and activates mitochondria. Finally, the protein enters the cell nuclei, where it accumulates.

The applicants have found that resistant and sensitive cells bind HAMLET to their surface with similar efficiency, suggesting that this is not the discriminating event. The nuclear accumulation, in contrast, occurs only in dying cells, suggesting that this step distinguishes sensitive tumour cells from resistant cells. By confocal microscopy, the nuclear accumulation appeared irreversible, suggesting the presence of nuclear targets that bind and retain HAMLET in the nuclear compartment.

What has now been found however is that HAMLET also appears to have an inhibitory effect on angiogenesis, which is believed to be greater than would be expected simply from the tumour killing activity previously noted. This is unexpected in view of the highly selective nature of the cellular effects of the molecule.

As a result, it increases the potential therapeutic range of the complex.

According to the present invention there is provided a biologically active complex of α-lactalbumin, selected from HAMLET or a biologically active modification thereof, or a biologically active fragment of either of these, in the preparation of a medicament for inhibiting angiogenesis.

Such medicaments can be used for treating cancers, and in particular solid cancers, and particularly rapidly proliferating solid tumors. In addition, however, it can be administered systemically to slow tumour metastasis.

The mechanism by which HAMLET or a biologically active modification thereof achieves this result is not understood. It may be expected that some effects would be mediated by tumour cells. Specifically, as HAMLET kills tumour cells, the supply of angiogenesis activator molecules is reduced. However, the effects noted appear to indicate that additional effects are occurring. For instance, it seems possible that HAMLET has a direct effect on rapidly proliferating vascular cells

It may also be used to treat other diseases where angiogenesis inhibition is desirable.

As a largely naturally occurring protein, it is believed that HAMLET will suffer from lower toxicity than entirely synthetic drugs. Furthermore, the immunogenicity of the complex is believed to be low.

The α-lactalbumin may be from various mammalian sources including human, bovine, sheep and goat milk, but is preferably human or bovine, and most preferably human. Recombinant forms of the α-lactalbumin may also be employed in the preparation of the biologically active complex.

It has also been found that other reagents and specifically lipids such as oleic acid, are useful in the conversion α-lactalbumin human α-lactalbumin to HAMLET. In particular, it has been reported previously that oleic acid (C18:1:9cis) is required for HAMLET production (M. Svensson, et al., (2000) *Proc Natl Acad Sci* USA, **97**, 4221-6). More recently, it has been found that other fatty acids may act as co-factors in a similar way. Optimal cofactors for the conversion of α-lactalbumin to HAMLET are C18:1 fatty acids with a double bond in the cis conformation at position 9 or 11.

α-Lactalbumin is a 14.2 kDa globular protein with four α-helices (residues 1-34, 86-123) and an anti-parallel β-sheet (residues 38-82), linked by four disulphide bonds (61-77; 73-91; 28-111 and 6-120) (K. R. Acharya, et al., (1991) *J Mol Biol,* **221**, 571-81). The native conformation of α-lactalbumin is defined by a high affinity Ca^{2+} binding site, co-ordinated by the side chain carboxylates of Asp82, Asp87 and Asp88, the carbonyl oxygens of Lys79 and Asp84, and two water molecules (K. R. Acharya, et al., (1991) *J Mol Biol,* **221**, 571-81). The protein adopts the so called apo-conformation found in HAMLET when exposed to low pH, or in the presence of chelators, that release the strongly bound Ca^{2+} ion (D. A. Dolgikh, et al., (1981) *FEBS Lett,* **136**, 311-5; K. Kuwajima, (1996) *Faseb J,* **10**, 102-09).

In order to form biologically active complexes, α-lactalbumin generally requires both a conformational or folding change as well as the presence of a lipid cofactor. The conformational change is suitably effected by removing calcium ions from α-lactalbumin. In a preferred embodiment, this is suitably facilitated using a variant of α-lactalbumin which does not have a functional calcium binding site.

Biologically active complexes which contain such variants are encompassed by the term "modifications" of HAMLET as used herein. However, the applicants have found that, once formed,

the presence of a functional calcium binding site, and/or the presence of calcium, does not affect stability or the biological activity of the complex. Biologically active complexes have been found to retain affinity for calcium, without loss of activity. Therefore complex of the invention may further comprise calcium ions.

Thus in particular, the invention uses a biologically active complex comprising alpha-lactalbumin or a variant of alpha-lactalbumin which is in the apo folding state, or a fragment of either of any of these, and a cofactor which stabilises the complex in a biologically active form, provided that any fragment of alpha-lactalbumin or a variant thereof comprises a region corresponding to the region of α-lactalbumin which forms the interface between the alpha and beta domains.

Suitably the cofactor is a cis C18:1:9 or C18:1:11 fatty acid or a different fatty acid with a similar configuration.

In a particular convenient embodiment, the biologically active complex used in the invention comprises
(i) a cis C18:1:9 or C18:1:11 fatty acid or a different fatty acid with a similar configuration; and
(ii) α-lactalbumin from which calcium ions have been removed, or a variant of α-lactalbumin from which calcium ions have been released or which does not have a functional calcium binding site; or a fragment of either of any of these, provided that any fragment comprises a region corresponding to the region of α-lactalbumin which forms the interface between the alpha and beta domains.

As used herein the expression "variant" refers to polypeptides or proteins which are homologous to the basic protein, which is suitably human or bovine α-lactalbumin, but which differ from the base sequence from which they are derived in that one or more amino acids within the sequence are substituted for other amino acids. Amino acid substitutions may be regarded as

"conservative" where an amino acid is replaced with a different amino acid with broadly similar properties. Non-conservative substitutions are where amino acids are replaced with amino acids of a different type. Broadly speaking, fewer non-conservative substitutions will be possible without altering the biological activity of the polypeptide. Suitably variants will be at least 60% identical, preferably at least 70%, even more preferably 80% or 85% and, especially preferred are 90%, 95% or 98% or more identity.

When comparing amino acid sequences for the purposes of determining the degree of identity, programs such as BESTFIT and GAP (both from Wisconsin Genetics Computer Group (GCG) software package). BESTFIT, for example, compares two sequences and produces an optimal alignment of the most similar segments. GAP enables sequences to be aligned along their whole length and finds the optimal alignment by inserting spaces in either sequence as appropriate. Suitably, in the context of the present invention when discussing identity of sequences, the comparison is made by alignment of the sequences along their whole length.

The term "fragment thereof" refers to any portion of the given amino acid sequence which will form a complex with the similar activity to complexes including the complete α-lactalbumin amino acid sequence. Fragments may comprise more than one portion from within the full length protein, joined together. Portions will suitably comprise at least 5 and preferably at least 10 consecutive amino acids from the basic sequence.
Suitable fragments will be deletion mutants suitably comprise at least 20 amino acids, and more preferably at least 100 amino acids in length. They include small regions from the protein or combinations of these.

The region which forms the interface between the alpha and beta domains is, in human α-lactalbumin, defined by amino acids 34-38 and 82-86 in the structure. Thus suitable fragments will

include these regions, and preferably the entire region from amino acid 34-86 of the native protein.

In a particularly preferred embodiment, the biologically active complex comprises a variant of α-lactalbumin in which the calcium binding site has been modified so that the affinity for calcium is reduced, or it is no longer functional.

It has been found that in bovine α-lactalbumin, the calcium binding site is coordinated by the residues K79, D82, D84, D87 and D88. Thus modification of this site or its equivalent in non-bovine α-lactalbumin, for example by removing one of more of the acidic residues, can reduce the affinity of the site for calcium, or eliminate the function completely and mutants of this type are a preferred aspect of the invention.

The Ca^{2+}-binding site of bovine α-lactalbumin consists of a 3_{10} helix and an α-helix with a short turn region separating the two helices (Acharya K. R., et al., (1991) *J Mol Biol* **221**, 571-581). It is flanked by two disulfide bridges making this part of the molecule fairly inflexible. Five of the seven oxygen groups that co-ordinate the Ca^{2+} are contributed by the side chain carboxylates of Asp82, 87 and 88 or carbonyl oxygen's of Lys79 and Asp84. Two water molecules supply the remaining two oxygen's (Acharya K. R., et al., (1991) *J Mol Biol* **221**, 571-581).

Site directed mutagenesis of the aspartic acid at position 87 to alanine (D87A) has previously been shown to inactivate the strong calcium-binding site (Anderson P. J., et al., (1997) *Biochemistry* **36**, 11648-11654) and the mutant proteins adopted the apo- conformation.

Therefore in a particular embodiment, the aspartic acid residue at amino acid position 87 within the bovine α-lactalbumin protein sequence is mutated to a non-acidic residue, and in particular a non-polar or uncharged polar side chain.

Non-polar side chains include alanine, glycine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan or cysteine. A particularly preferred examples is alanine. Uncharged polar side chains include asparagine, glutamine, serine, threonine or tyrosine.

In order to minimize the structural distortion in the mutant protein, D87 has also been replaced by an asparagine (N) (Permyakov S. E., et al., (2001) *Proteins Eng* **14**, 785-789), which lacks the non-compensated negative charge of a carboxylate group, but has the same side chain volume and geometry. The mutant protein (D87N) was shown to bind calcium with low affinity ($K\text{-}_{Ca}2 \times 10^5 M^{-1}$) (Permyakov S. E., et al., (2001) *Proteins Eng* **14**, 785-789). Such a mutant forms an element of the biologically active complex in a further preferred embodiment of the invention.

Thus particularly preferred variants for use in the complexes of the invention are D87A and D87N variants of α-lactalbumin, or fragments which include this mutation.

This region of the molecule differs between the bovine and the human proteins, in that one of the three basic amino acids (R70) is changed to S70 in bovine α-lactalbumin thus eliminating one co-ordinating side chain. It may be preferable therefore, that where the bovine α-lactalbumin is used in the complex of the invention, an S70R mutant is used.

The Ca^{2+} binding site is 100% conserved in α-lactalbumin from different species (Acharya K. R., et al., (1991) *J Mol Biol* **221**, 571-581), illustrating the importance of this function for the protein. It is co-ordinated by five different amino acids and two water molecules. The side chain carboxylate of D87 together with D88 initially dock the calcium ion into the cation-binding region, and form internal hydrogen bonds that stabilise the structure (Anderson P. J., et al., (1997) *Biochemistry* **36**,

11648-11654). A loss of either D87 or D88 has been shown to impair Ca2+ binding, and to render the molecule stable in the partially unfolded state (Anderson P. J., et al., (1997) *Biochemistry* **36**, 11648-11654).

Further, mutant proteins with two different point mutations in the calcium-binding site of bovine α-lactalbumin may be used. For example, substitution of the aspartic acid at position 87 by an alanine (D87A) has been found to totally abolish calcium binding and disrupt the tertiary structure of the protein. Substitution of the aspartic acid by asparagine, the protein (D87N) still bound calcium but with lower affinity and showed a loss of tertiary structure, although not as pronounced as for the D87A mutant (Permyakov S. E., et al., (2001) *Proteins Eng* **14**, 785-789). The mutant protein showed a minimal change in packing volume as both amino acids have the same average volume of 125Å^3, and the carboxylate side chain of asparagines allow the protein to co-ordinate calcium, but less efficiently (Permyakov S. E., et al., (2001) *Proteins Eng* **14**, 785-789). Both mutant proteins were stable in the apo-conformation at physiologic temperatures but despite this conformational change they were biologically inactive. The results demonstrate that a conformational change to the apo-conformation alone is not sufficient to induce biological activity.

The structure of α-lactalbumin is known in the art, and the precise amino acid numbering of the residues referred to herein can be identified by reference to the structures shown for example in Anderson et al. supra. and Permyakov et al supra.

The medicaments produced in accordance with the invention are suitably pharmaceutical compositions in a form suitable for topical use, for example as creams, ointments, gels, or aqueous or oily solutions or suspensions. These may include the commonly known carriers, fillers and/or expedients, which are pharmaceutically acceptable.

Topical solutions or creams suitably contain an emulsifying agent for the protein complex together with a diluent or cream base.

The daily dose of the active compound varies and is dependant on the patient, the nature of the condition being treated etc. in accordance with normal clinical practice. As a general rule from 2 to 200 mg/dose of the biologically active complex is used for each administration.

In a further aspect of the invention, there is provided a method for inhibiting angiogenesis which comprises administering to a patient in need thereof, a biologically active complex of α-lactalbumin, selected from HAMLET or a biologically active modification thereof, or a biologically active fragment of either of these.

Preferred examples of the biologically active complex are illustrated above. Preferably the biologically active complex is administered in the form of a topical composition, also as described above.

The invention will now be particularly described by way of example, with reference to the accompanying drawings in which Figures 1 to 3 show a progressively enlarged tissue section illustrating the disassembly of blood vessels in a human bladder papilloma after topical HAMLET treatment for 5 days.

Example 1

Intra-Vesical Instillation Of Hamlet In Patients With Cancer Of The Urinary Bladder

Preparation of substance and randomisation of patients

Donors of breastmilk were non-smokers and were screened for HIV prior to preparation of HAMLET. Alpha-lactalbumin was purified from human milk whey by ammonium sulphate precipitation followed by phenyl-Sepharose chromatography and size-exclusion chromatography. Excess milk from the hospital milk bank was used according to regulations for administration to premature

babies. HAMLET was generated from native α-lactalbumin on an oleic acid conditioned ion-exchange chromatography column, as described in the literature. The eluted fractions were dialysed against distilled water, lyophilised and stored at -20°C.

Furthermore, HAMLET was screened for bacterial contamination and was stored as dry substance in -20°C.

Study design:

Patients awaiting surgery for a newly diagnosed, or recurrent uro-epithelial cancer of the urinary bladder, were invited to participate in the study. After informed consent the patients were subjected to cystoscopy to assess the tumour size and to document the lesion with endoluminal photography. After treatment and prior to surgery, cystoscopy was repeated to re-assess tumour size and endoluminal photography was carried out.

Intra-vesical instillation of HAMLET was performed in the out-patient clinic under close surveillance. The instillations were given once daily, and repeated for five days. After urethral catheterisation the bladder was completely emptied and the urine was collected for analysis. HAMLET (25mg/ml, 30ml) was deposited in the bladder, the catheter removed, and the patients were asked to too keep the instillation for at least for two hours. To decrease the diuresis the patients were asked to avoid fluid intake for four hours before, and immediately after the instillation. Urine samples were provided prior to, and from the first voided urine after each instillation.

A biopsy sample of a treated tumour was taken at the end of this treatment, and the results are shown in Figure 1 to 3. As is clear from these figures, the endothelial lining is missing and blood corpuscles are present throughout the core of the tumor, indicating that angiogenesis has been inhibited.

Claims

1. The use of a biologically active complex of α-lactalbumin, selected from HAMLET or a biologically active modification thereof, or a biologically active fragment of either of these, in the preparation of a medicament for use in the inhibition of angiogenesis.

2. The use according to claim 1 wherein the biologically active complex comprising alpha-lactalbumin or a variant of alpha-lactalbumin which is in the apo folding state, or a fragment of either of any of these, and a cofactor which stabilises the complex in a biologically active form, provided that any fragment of alpha-lactalbumin or a variant thereof comprises a region corresponding to the region of α-lactalbumin which forms the interface between the alpha and beta domains.

3. The use according to claim 2 wherein the cofactor is a cis C18:1:9 or C18:1:11 fatty acid or a different fatty acid with a similar configuration.

4. The use according to any one of claims 1 to 3 wherein the biologically active complex comprises HAMLET, which is obtainable either by isolation from casein fractions of milk which have been precipitated at pH 4.6, by a combination of anion exchange and gel chromatography, or by subjecting α-lactalbumin to ion exchange chromatography in the presence of a cofactor from human milk casein, characterized as C18:1 fatty acid.

5. The use according to any one of claims 1 to 3 wherein the biologically active complex of α-lactalbumin comprises (i) a cis C18:1:9 or C18:1:11 fatty acid or a different fatty acid with a similar configuration; and

(ii) α-lactalbumin from which calcium ions have been removed, or a variant of α-lactalbumin from which calcium ions have been removed or which does not have a functional calcium binding site; or a fragment of either of any of these, provided that any fragment comprises a region corresponding to the region of α-lactalbumin which forms the interface between the alpha and beta domains.

6. The use according to claim 5 wherein the biologically active complex includes a variant of α-lactalbumin in which the calcium binding site has been modified so that the affinity for calcium is reduced, or it is no longer functional.

7. The use according to claim 6 wherein the variant has a mutation at one of the amino acids equivalent to K79, D82, D84, D87 and D88 of bovine α-lactalbumin.

8. The use according to claim 7 wherein the modification is at D87 which includes a variant of α-lactalbumin having a D87A or D87N variants.

9. The use according to any one of claims 1 to 3 wherein the biologically active complex comprises a fragment of α-lactalbumin or a variant thereof, and where the fragment includes the entire region from amino acid 34-86 of the native protein.

10. The use according to any one of the preceding claims wherein the α-lactalbumin is human or bovine α-lactalbumin or a variant of either of these.

11. The use according to claim 10 wherein the α-lactalbumin is human α-lactalbumin.

12. The use according to claim 10 wherein the α-lactalbumin is mutant bovine α-lactalbumin which includes an S70R mutation.

13. The use according to any one of the preceding claims which is for slowing metastasis of tumour cells.

14. A method for inhibiting angiogenesis which comprises administering to a patient in need thereof, a biologically active complex of α-lactalbumin, selected from HAMLET or a biologically active modification thereof, or a biologically active fragment of either of these.

Abstract

Novel Therapies

The use of a biologically active complex of α-lactalbumin, selected from HAMLET or a biologically active modification thereof, or a biologically active fragment of either of these, in the preparation of a medicament for use in the inhibition of angiogenesis. Such medicaments may be used in the treatment of various conditions where inhibition of angiogenesis is desirable, including the treatment of malignant or non-malignant tumours, as well in slowing tumour metastasis.

Figure 1

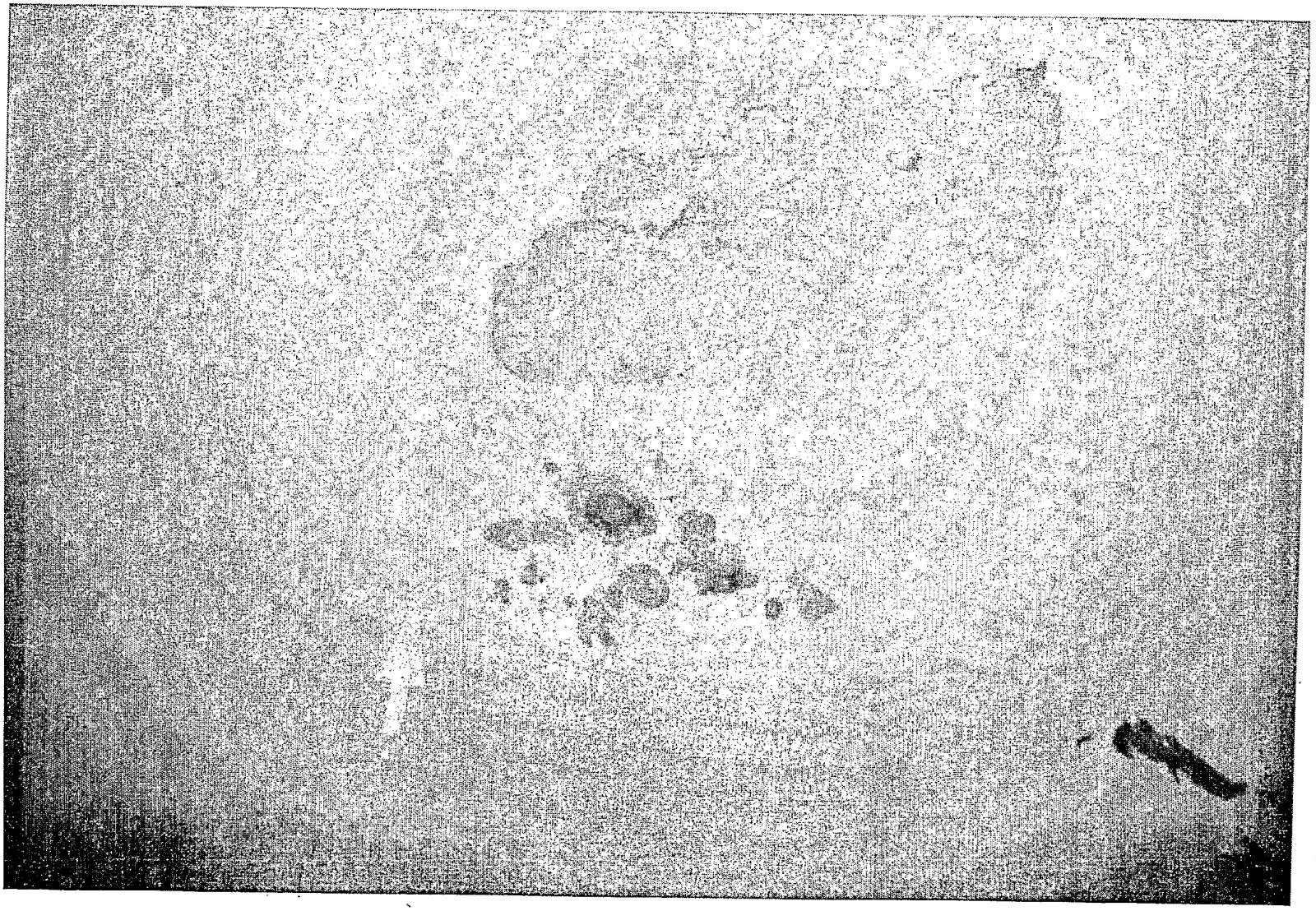

Figure 2

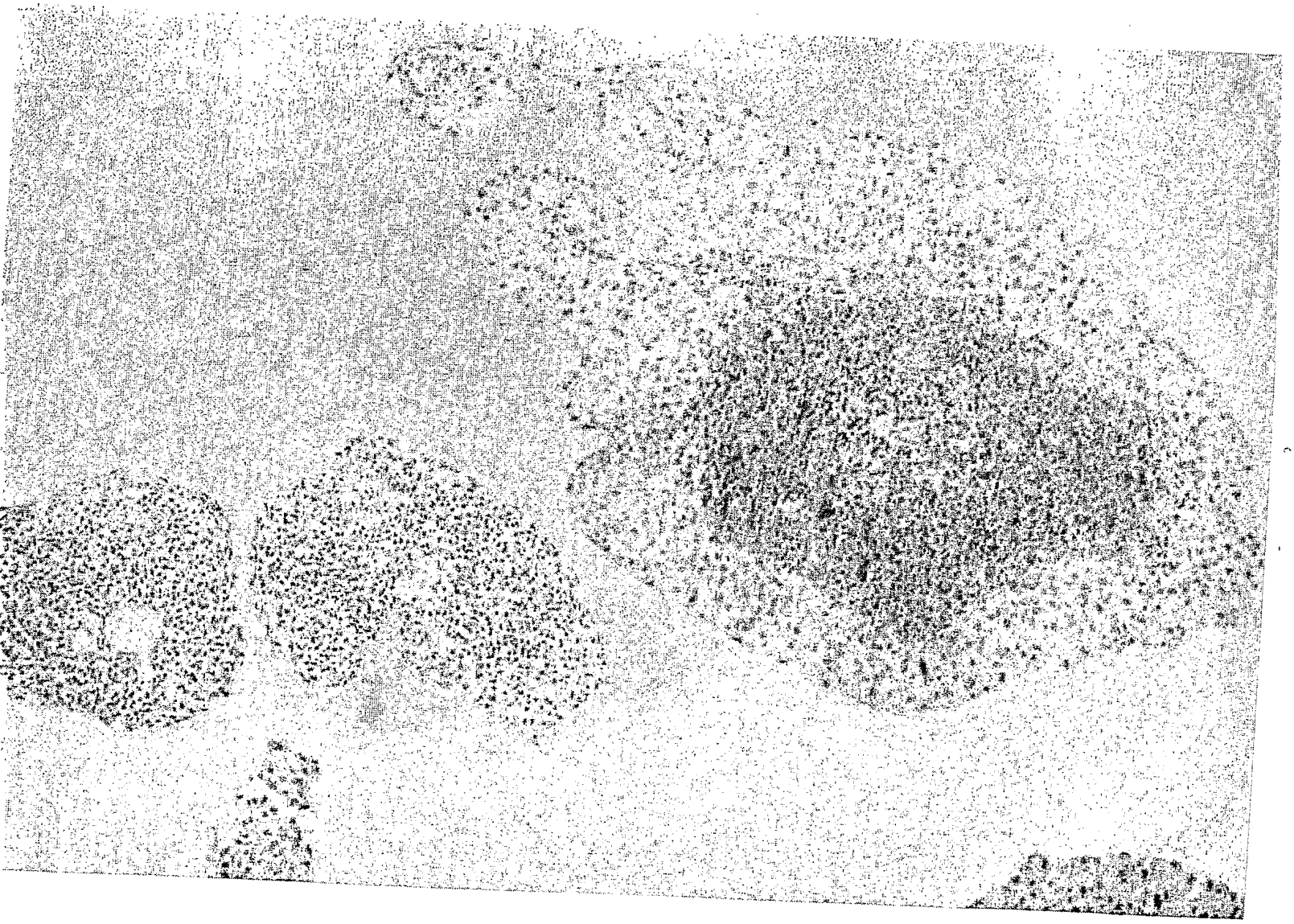

Figure 3